TOXINS AND THE SIDE-CHAIN THEORY*

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The toxins are distinguished from other poisonous agents by their property of stimulating the production of antibodies (antitoxins) when they are injected into animals. While, previous to the studies on cobra venom, nothing had been known regarding the mechanism of the action of toxins, that action had found an attempted explanation in the widely accepted side-chain theory of Ehrlich,[1] which considers not only the poisonous action of toxins, but also the mechanism of antitoxin production.

According to the side-chain theory, the toxin molecule is conceived to possess a chemical group by which it is capable of entering into firm[1] chemical union, through a corresponding chemical group, with the sensitive tissue. The uniting group of the toxin molecule has been named the "haptophore" group and that of the vulnerable cell, "receptor." The union of toxin and cell having taken place—but not till then—the toxin is able to inflict its characteristic injury upon the cell, that injury being produced by a second chemical group, the so-called toxophore group. The tissue receptors that have united with the introduced toxin are assumed to have been thrown out of function in the cell economy and this elimination of the "receptors" is supposed to constitute in itself an injury that must be repaired. The injury caused by the mere union of the "haptophore" group with the cell receptors is different from that subsequently inflicted by the "toxophore" group, since the former injury must also be assumed, under the side-chain theory, to follow the injection of non-toxic antigenic substances, such as foreign blood sera or corpuscles. In repairing the defect, the cell is believed to produce a greater number of the "receptors" than were used up by the toxin. The excessive "receptors" are thrown off into the body fluids and constitute there the specific antibodies, or antitoxin. In explaining this excessive production of the "receptors," Ehrlich has cited the analogous excessive production of tissue in the repair of the

* Received for publication June 8, 1915.

1. Proc. Roy. Soc., 1900, 66, p. 424.

loss of substance following injuries. This parallel clearly expresses the conception that the union of haptophore group and cell receptor results in injury to the cell, for there is in nature no known instance of a loss of tissue not accompanied with injury that is followed by an overproduction of that tissue.

It should be remarked here in passing that the laws governing the response of the cells to antigenic stimulation are quite different from those controlling the response of tissues to the effect of injurious agents; that is, to irritants. It is a fact of common knowledge that if a tissue be exposed at intervals to the influence of an irritant, that tissue becomes increasingly resistant to the irritant and the cell multiplication, which is the index of reaction on the part of the tissue, becomes less and less. In their discussion of this point, von Dungern and Werner[2] write:

> If a definite irritation be repeated at intervals of sufficient length, so that in the interim the cells may return to the resting state, an important phenomenon will be observed. The reaction to the irritant becomes less and less, until finally the same degree of irritation that at first set up a vigorous growth is no longer able to produce any increase in proliferation. If an irritant be applied continuously or at very short intervals, the end result is different according to the intensity of the irritation. If the latter be mild, an increased power of resistance against the growth-producing irritant takes place, so that the proliferation not only does not progress, but at last actually diminishes. If, on the other hand, the intensity of the irritant is great, the recovery of the cells from the injury is prevented, and, as a result of a cumulative action of the irritant, an increase of growth takes place up to a definite maximum, to be followed, upon further irritation, by the death of the cell.

On the other hand, tissues that are exposed at intervals to antigenic stimulation exhibit, at first, more and more sensitiveness to that stimulation, as is shown by the increased production of the specific antibodies, up to a certain height, after which, instead of a power of resistance, or hyposensitiveness, developing against the antigenic stimulation, the response may continue unabated for an indefinite period, subject only to the condition of the general health, which, it must be remembered, may be affected by the injections of the antigen.

The effect of repeated injections carried out over an extended period has been exactly studied by Elser and Huntoon, to whom I am greatly indebted for permission to refer to some of those studies in advance of their final publication: Four rabbits, 441, 234, 286, and 285, received daily intravenous injections of the same small amount of

2. Das Wesen der bösartigen Geschwülste., 1907.

meningococci over periods of 13 months, 12 months, 8 months, and 36 months, respectively (the last animal receiving 1091 injections). In all of these animals, the rate of antibody production increased rapidly, at first, up to a maximum, after which, with the exception of certain fluctuations of greater or less extent, which often could be referred to conditions affecting the general health of the animal, such as pregnancy and infection, the rate remained relatively high. In no instance was a decline observed in the rate of antibody production that could be referred to a repetition of the injections themselves. Two other rabbits that received similar injections of typhoid bacilli over a period of three months responded in precisely the same way.

Two rabbits that I have treated in the same manner with sheep blood corpuscles (0.1 c.c. of blood daily) yielded a hemolytic serum of a strength of 1 : 40,000, which was maintained practically unchanged for a period of several weeks.

It is seen that the reaction of the tissues to antigenic stimulation is different from that of tissues to injurious irritation. This antithesis, which hitherto has been overlooked, cannot be compromised; it prevents the application of the principle of Weigert to the explanation of the mechanism of antibody production. The reaction of the tissues to antigenic stimulation finds a more plausible physiologic analogy in the response of digestive glands to specific chemical stimulation offered by foodstuffs than in the response of tissues to injurious influences.

The toxins bear a long-recognized, striking resemblance to the organic ferments. This resemblance, however, has not led to any general belief in the fermentative nature of the toxins. On the contrary, the only writer on this question in recent times, von Liebermann,[3] emphatically denied that the toxins are ferments. Moreover, von Liebermann made the noteworthy remark that the establishing of the fermentative nature of the toxins would render the side-chain theory untenable. Von Liebermann based his denial of the fermentative nature of the toxins upon the results of his experiments with the agglutinating property of ricin and abrin. Those results are as follows: (1) The agglutinating principle of these preparations is quickly absorbed in a definite quantitative proportion by the blood corpuscles; that is, these "toxic" bodies are "used up" in the reaction, whereas the ferments, theoretically, are not used up. (2) The ricin agglutinin is

3. Deutsch. med. Wchnschr., 1905, 31, p. 1301.

not appreciably affected by hydrocyanic acid, which is a "violent enzyme poison." (3) The agglutinating property of ricin is not destroyed by exposure for half an hour to a temperature of from 70 to 80 C.; that is, this "toxic" body is not thermolabile as are the ferments. Von Liebermann recognized that the incompleteness of our knowledge about ferments could be urged against drawing any deductions regarding the fermentative nature of toxins, but believed nevertheless that that knowledge was sufficient to support the evidence on which he based his denial. However, it is just at that point that his whole argument has fallen; for since he published these views a ferment has been found that is absorbed by the substratum upon which it acts (appearing thus to have been used up), that is not appreciably affected, as we shall show, by hydrocyanic acid, and that is not affected by exposure for half an hour to a temperature of over 70 C. The ferment referred to is the lipase of cobra venom, which is also the hemotoxin of the venom.

The absorption of the lipase of cobra venom by lecithin was first demonstrated by Kyes and amply confirmed by von Dungern and Coca, and Manwaring. Von Dungern and Coca showed that the absorbed lipase was not actually used up, but could be demonstrated in active condition in the digestion products of the lecithin, and later Manwaring was able to isolate the lipase again from those digestion products.

Therefore, the fact that a toxin is absorbed by the vulnerable tissue does not show that the toxin is used up in the process of its activity; hence, its ferment nature cannot be disputed on this ground.

We have investigated the influence of hydrocyanic acid upon the lipase of cobra venom and have found that the lipolytic action of the ferment is not interfered with by this acid.

Six and one-half grams of potassium cyanid (Kahlbaum, 96-98 percent pure) were mixed with 47 c.c. of strong HCl (10 c.c.-2.9 c.c. normal), and the final volume of the mixture was then brought up to 50 c.c. by the addition of distilled water. The resulting fluid thus contained about 5.2 percent of hydrocyanic acid and 15 percent of potassium chlorid. The reaction of the fluid was very slightly alkaline to litmus, and twenty drops of it caused no hemolysis of 1 c.c. of a 5 percent suspension of ox blood corpuscles.

Four dilutions of cobra venom (for which I am under obligation to Prof. A. Calmette, director of the Pasteur Institute in Lille), were prepared containing, respectively, 1 in 10,000, 1 in 100,000, 1 in 1,000,000, and 1 in 10,000,000 of the venom. Three series of twelve tubes each were arranged, each series containing 1 c.c., 0.5 c.c., and 0.2 c.c. of the four venom dilutions, and each tube containing 0.1 c.c. of a 0.2 percent emulsion of lecithin, which by itself was not hemolytic in a quantity of 0.4 c.c.

To each tube of Series A was added 1 c.c. of the hydrocyanic acid solution (von Liebermann used 0.2 c.c. of a 72 percent solution) and to each tube of Series B was added an equal volume of a 15 percent solution of potassium chlorid. No addition was made to the third series, C.

After these three series had stood for twenty-four hours at room temperature, 1 c.c. of a 5 percent suspension of ox blood was added to each tube and the hemolysis resulting at room temperature was observed. In the first three tubes of Series C, hemolysis was complete in a few minutes, whereas in the corresponding tubes of Series A and B complete solution occurred much later (from one to three hours). At the end of a further twenty-four hours, the tube of Series C containing 1 c.c. of the fourth dilution showed complete hemolysis; that containing 0.5 c.c. of the fourth dilution showed strong hemolysis; and that containing 0.2 c.c. of the fourth dilution showed a trace of hemolysis. In Series A, the corresponding tubes showed complete hemolysis, slight hemolysis, and a trace of hemolysis. In Series B, the corresponding tubes showed strong hemolysis, no hemolysis, and no hemolysis. It is seen that not even during twenty-four hours contact was the hydrocyanic acid able appreciably to interfere with the lipolytic activity of the venom.

The relative resistance of the cobra venom lipase to heat is already known. Heating a 1 percent solution of the venom for three quarters of an hour at 72 C. does not noticeably affect its lipolytic activity, which is undiminished even by short boiling.

All of the arguments brought by von Liebermann against the theory of the fermentative nature of the toxins are thus seen to have lost their force through the subsequent studies on the lipase of cobra venom. Moreover, these same studies have demonstrated that one toxin (the hemotoxin of the venom) is actually a ferment; in other words, the hemotoxin has been shown to be identical with the lipase of the venom.

The hemotoxin of cobra venom is that constituent of the venom which destroys red blood corpuscles. Not all species of red blood corpuscles are susceptible to the direct action of the hemotoxin; those of the guinea-pig and man, for example, being very susceptible, and those of the ox and the sheep being non-susceptible. The red blood corpuscles that are invulnerable to the direct action of the hemotoxin can be made vulnerable with the co-operation of certain "activators," such as normal serum and lecithin. The mechanism of the "activation" is not the same with these two agents. The destruction of the corpuscles is brought about in the two cases through the action of two different constituents of the venom. This is shown by the fact that, on the one hand, treatment of the normal serum in the cold with the invulnerable corpuscles greatly diminishes the activating power of the serum, and that, on the other hand, heating the venom solution in an

acid medium causes it to lose its power of destroying the invulnerable corpuscles when it is mixed with normal serum, altho it is still able to do so in co-operation with lecithin.

The present discussion concerns itself only with the venom constituent that attacks the naturally invulnerable corpuscles with the aid of lecithin. The researches of Kyes, Kyes and Sachs, von Dungern and Coca, and Manwaring have shown that the venom-lecithin hemolysis is brought about by the fermentative action of the venom on the lecithin whereby the latter, a non-hemolytic substance, is split into two parts both of which are strongly hemolytic. These two parts are oleic acid and the lecithin rest, which is lecithin in which one of the two fatty acid molecules is missing. Because of the demonstrated fact that this mono-fatty acid lecithin rest contains no oleic acid, it has been designated "desoleolecithin." The properties by which this substance is recognized are, its solubility in alcohol and water, its insolubility in ether, and its great hemolytic power.

It has been assumed that the mechanism of the direct hemolytic action of the cobra hemotoxin in destroying naturally vulnerable corpuscles is the same as that of its indirect hemolytic action in co-operation with lecithin. The immediate hemolytic agents in the former case would thus be the split products of the lecithin that is normally present in the red blood corpuscles in a quantity sufficient to supply a completely hemolytic amount of desoleolecithin and oleic acid. In accordance with this assumption, it was conceivable that desoleolecithin could be demonstrated in the fluid resulting from the direct hemolytic action of the venom on naturally vulnerable corpuscles. The following experiments will show that such demonstration is possible.

A preliminary experiment was carried out in order to find out what part of a definite quantity of desoleolecithin that has been mixed with blood corpuscles can be recovered with the method at our disposal.

One hundred cubic centimeters of ox blood were well washed with physiologic salt solution and the corpuscular sediment was mixed with 0.2 gm. of desoleolecithin (two complete hemolytic doses) which had been dissolved in salt solution. (The minimal completely hemolytic quantity of this preparation for 1 c.c. of 5 percent ox blood was 1/20,000 gm.) After the resulting hemolysis was complete, 200 c.c. of distilled water and 100 c.c. of 95 percent alcohol were added and the mixture was boiled for a few minutes. The fluid, which will be referred to as the "first extract," was separated from the coagulated proteins by filtration. The coagulated proteins were mixed with 400 c.c. of 95 percent alcohol and allowed to stand over night at room temperature.

The first extract was evaporated at first by boiling and finally with the use of an electric fan, and the residue was extracted with ether. The ether extract

was discarded and the residue was again extracted with warm absolute alcohol. The alcoholic extract was evaporated with the use of the electric fan, and the residue was taken up in 20 c.c. of physiologic salt solution. One cubic centimeter of a 5 percent suspension of washed ox blood was completely hemolysed in a few minutes by 0.4 c.c. of this solution and slightly dissolved by 0.2 c.c. of the solution. The entire 20 c.c. therefore contained enough of the hemolysin to dissolve completely 2.5 c.c. of undiluted ox blood.

The second alcoholic extract of the coagulated proteins of the ox blood was obtained by filtration after the mixture of the proteins and after the alcohol had stood over night at room temperature. The filtrate was evaporated to dryness with the use of the electric fan, and the residue thus obtained was extracted with ether, the ethereal extract being discarded. The residue was then extracted with warm absolute alcohol and the alcoholic extract, separated by centrifugation, was evaporated to dryness and the resulting residue taken up in 10 c.c. of warm physiologic salt solution. This solution hemolysed 1 c.c. of 5 percent ox blood in a minimal quantity of 0.02 c.c., and the entire 10 c.c. contained, therefore, enough of the hemolysin to dissolve completely 12.5 c.c. of undiluted ox blood.

The hemolysin obtained by the procedure just described was identified as desoleolecithin, first, by its solubility in alcohol and water, and its insolubility in ether, which distinguishes it from the hemolytic substances in the extracts of normal tissue; secondly, by the rapidity of its hemolytic action, the minimal dose producing complete hemolysis within a few minutes; and finally, by reason of the failure of this method of extraction, in several experiments, to demonstrate any such hemolysin in corpuscles that had not been dissolved either with cobra venom or desoleolecithin. The experiment therefore demonstrates that even if a quantity of desoleolecithin capable of completely dissolving 200 c.c. of undiluted ox blood be mixed with only 100 c.c. of such blood, it is possible, with the method of extraction used, to recover from the hemolyzed corpuscles only about 7.5 percent of the desoleolecithin taken. By far the greater part of the hemolysin was combined with the corpuscular substance in such a way that the method of extraction employed failed to separate it.

The same method of extraction was applied to guinea-pig blood corpuscles that had been hemolyzed either with distilled water or with native cobra venom. In the former instance no hemolytic substances at all were obtained, but in the latter instance a quantity of hemolysin having the properties of desoleolecithin previously mentioned was obtained that was equivalent to about 2.5 percent of the calculated minimal amount necessary completely to dissolve the corpuscles used.

For this experiment 400 c.c. of defibrinated guinea-pig blood were well washed with physiologic salt solution and the corpuscular sediment was divided into two equal portions. To one portion was added 0.1 gm. of cobra

venom dissolved in 5 c.c. of physiologic salt solution. After three quarters of an hour at room temperature, microscopic examination showed that the corpuscular forms had completely disappeared and that the thick fluid contained masses of hemoglobin crystals. At the end of two hours, 100 c.c. of distilled water were added to each portion of blood and both portions were treated according to the method of extraction previously described. Thirty cubic centimeters of the final salt solution extract were obtained from each portion. That derived from the blood that had been dissolved with distilled water lacked completely any hemolytic power; that derived from the portion of blood that had been dissolved with cobra venom was completely hemolytic in a minimal quantity of 0.3 c.c.

The entire amount of the extract thus contained enough of the hemolysin to dissolve 100 c.c. of 5 percent guinea-pig blood, or 5 c.c. of undiluted blood, which is 2.5 percent of the quantity in each portion of blood used for the experiment.

A similar experiment, the notes of which have been lost, led to the same result; a considerable quantity of the hemolysin possessing the properties of desoleolecithin was again extracted from guinea-pig corpuscles that had been dissolved by the direct action of cobra venom.

The same method of extraction was applied also to ox blood corpuscles that had been dissolved by the combined action of anti-ox corpuscle amboceptor serum and normal guinea-pig serum, with negative result, the method of extraction failing to discover the production in the dissolved corpuscles of any hemolytic substance possessing the properties of desoleolecithin.

The sediment of 200 c.c. of well-washed ox blood was mixed with 25 c.c. of the serum of a rabbit that had received injections of ox blood, and to this mixture were then added 200 c.c. of normal guinea-pig serum. Two hours later, complete hemolysis having taken place in thirty-five minutes, the mixture was treated with the alcohol and ether extraction method. The final residue was taken up in 3 c.c. of salt solution, and this solution was then found to be entirely lacking in hemolytic activity when mixed with 1 c.c. of 5 percent ox blood.

The results of the experiments just described leave no room for doubt as to the mechanism of the direct destructive action of the cobra hemotoxin. It is clear that this hemolytic action is brought about by the lipolytic power of that constituent of the venom whereby the normally contained lecithin, and no doubt other fatty substances as well, are split into products that are the ultimate cell-destroying substances. It follows, therefore, that the cell destruction in this instance cannot be due to any assumed chemical group (toxophore group) in the toxin molecule.

Furthermore, if any of the toxin does attach itself to the cell proper according to the conception of the side-chain theory (through a haptophore group), as has been shown by von Dungern and Coca[4] to occur when naturally immune corpuscles (ox corpuscles) are brought into contact with a solution of cobra venom, the cell is not injured thereby. It is only when the toxin reaches the lecithin, becomes dissolved in it, and splits it into desoleolecithin and oleic acid, both of which are highly injurious to the cell, or when it reaches some other fatty substance and splits it into glycerin and some lower fatty acid, both of which are cell poisons—only then does the injurious influence of the hemotoxin reveal itself.

The two important results that have come out of the investigations of the hemotoxin of cobra venom are: First, the demonstration of the fermentative nature of a toxin, and second, the demonstrated failure of the side-chain theory in the first real test to which it has been put as an explanation of the mechanism of toxin action. In view of the first of these results, it seems safe to assume that all toxins may be ferments. The sources of the ferments and those of the toxins are the same. Ferments as well as toxins are known to exist in glandular secretions of animals and in the products of the growth of bacteria and in the higher forms of plant life. We have already recalled the long-recognized similarity between the general properties of ferments and those of toxins.

Another result of the investigations of the hemotoxin of cobra venom is the explanation of the natural resistance of the invulnerable corpuscles (ox and sheep) to the direct action of the hemotoxin. This natural resistance, or immunity, has been found to depend upon a physical condition of the cell substance that prevents the hemotoxin from penetrating to the lipoids of the corpuscles. This was shown by the discovery of Goebel[5] that the mere suspending of the naturally immune corpuscles in a chemically inert solution of sugar suffices so to alter the physical condition of the corpuscular substance that the hemotoxin can then enter the cells, reach the lipoids, and cause hemolysis. Other substances, also, such as soap and oleic acid, have been found to produce a physical alteration of the corpuscles that is similar in effect to that produced by the sugar.

As the discovery of the nature and manner of action of the hemotoxin of cobra venom has revealed the probable nature of the other

4. München. med. Wchnschr., 1907, 47, p. 2317.
5. Compt. rend. de Soc. de biol., 1905, 58, p. 422.

toxins, so also the explanation of the natural immunity of the invulnerable corpuscles to the direct action of the venom hemotoxin may provide a clue to the mechanism of some of the other known instances of natural immunity to toxins.

It is evident that the explanation offered by Ehrlich[1] for natural immunity cannot be brought into harmony with the demonstrated mechanism of the natural immunity of blood corpuscles to the direct action of the hemotoxin of cobra venom. Ehrlich's explanation was that the cells of the naturally immune animal do not possess receptors capable of entering into chemical union with the haptophore group of the respective toxin molecule. This explanation is incompatible with the demonstration of Goebel that the natural immunity of invulnerable corpuscles to the direct action of cobra hemotoxin is dependent upon a physical condition of the corpuscular substance.

Of the numerous cases of natural immunity to toxins that have been studied, only few are known (such as that of the scorpion to its own venom and that of the hedge-hog to the viper's venom) which could be referred to any antitoxic power of the blood. Indeed, some of the naturally immune animals are not capable of producing antitoxic substances, even after having received large injections of the toxin.

There are a few cases known in which a relative natural immunity is present so long as the body temperature of the animal remains below a certain point. The frog, in winter, and the hibernating bat and marmot possess such a relative resistance to tetanus toxin. At the higher "summer" temperature, all these animals become susceptible.

The remarkable natural immunity of the fowl against tetanus toxin remains an inexplicable phenomenon. The blood of the fowl is entirely lacking in antitoxic property; the immunity is therefore not humoral. Under ordinary condition of health, large subcutaneous or intraperitoneal injections of the toxin are borne by fowl without symptoms; but, if the injections are made into the brain or after the fowl have become weakened by cold, the toxin is able to produce its characteristic effect. It is conceivable that in this case, as well as in a number of other instances in which the immunity is demonstrably not humoral, the physical condition of some protective tissue or of the sensitive cells themselves plays a determining role.